Comparison of Bacterial Communities in Two Partial Nitrification Systems for High-ammonia Wastewater and Sewage Treatment

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ABSTRACT

Partial nitritation is an important part of the biological nitrogen removal processes; it saves half of the aeration energy, since only half of NH_4^+–N need to be oxidized to nitrite. The performance of the process was determined by the microbial community structure. In this study, we measured the microbial diversity in terms of the quantity of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) that were present. The results revealed that the amount of aerobic bacteria did not change significantly in high-ammonia wastewater, but decreased significantly with running time in sewage. The abundance of AOB and NOB in high-ammonia wastewater ranged from 1.23×10^7 to 8.95×10^8 copies/mL mixed liquor, and 1.11×10^4 to 2.47×10^6 copies/mL mixed liquor, respectively. These values were significantly higher than those in sewage (4.19×10^5 to 3.95×10^7 copies/mL mixed liquor and 1.46×10^3 to 5.89×10^5 copies/mL mixed liquor, respectively). Phylogenetic analysis showed that *Nitrosospira* was only found in high nitrogen wastewater, and that *Nitrosomonas* was the dominant bacterium in 2 kinds of wastewater, whereas the clusters of *Nitrosomonas* varied according to the nitrogen concentration of wastewater. Thus, the abundance and composition of AOB and NOB populations are significantly correlated with nitrogen concentration.

Keywords: Partial nitritation, ammonia oxidizing bacteria, nitrite oxidizing bacteria, denaturing gradient gel electrophoresis, quantitative PCR

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INTRODUCTION

Nitrogen can be removed from wastewater by a variety of physicochemical and biological processes, although biological processes are preferred since they are usually more environmental friendly (Tassadaq et al. 2013). Discovering an efficient biological way to reduce the operation costs of wastewater treatment plants is a major goal in the industry worldwide. Process revolution and process control can be used to resolve this problem with respect to mechanistic and operational aspects, respectively. The discovery of anaerobic ammonium oxidation (anammox) bacteria facilitates autotrophic partial nitritation, because anammox bacteria can directly convert nitrite and ammonium to nitrogen gas in the absence of an organic carbon source. This occurs in the partial nitritation phase, since only half of NH_4^+ needs to be oxidized to NO_2^- rather than NO_3^- (Zhang et al., 2001; Cébron et al., 2004).

This new type of biotechnological process has been successfully applied in the high-strength ammonium wastewater treatment. Meanwhile, its potential utility in low ammonium sewage treatment remains unclear (Kartal et al., 2010). Therefore, there is a need for further study of the ability of bacterial communities to convert NH_4^+ to NO_2^- in sewage wastewater via the process of partial nitritation.

Many studies have investigated the activity of nitrifying bacterial species. Gene sequence analysis has revealed that there are 5 genera of AOB: *Nitrososmonas, Nitrosococcus, Nitrosospira, Nitrosolobus*, and *Nitrosovibrio*; and 4 of NOB: *Nitrospira, Nitrobacter, Nitrospina*, and *Nitrococcus*. To explain the role of nitrifying bacterial communities in the treatment process, some researchers have assumed that AOB and NOB were divided into slow-growing or fast-growing groups. Slow-growing bacteria, referred to as K strategists, such as *Nitrosospira* and *Nitrospira*, have a high affinity for substrate and are dominant at low substrate concentrations. In contrast, fast-growing bacteria, referred to as R strategists, such as *Nitrosomonas* and *Nitrobacter*, have a low affinity for substrate and thrive at high substrate concentrations (Ma et al., 2011; Noguera et al., 2002). Painter et al. (Painter et al., 1986) found that *Nitrosomonas europa* were the main genera of AOB. In contrast, studies using 16S ribosomal RNA (rRNA)-targeted methods revealed a great diversity of nitrifiers in activated sludge. *Nitrosospira* were identified as the main genera of AOB in both bench-scale systems (Gieseke et al., 2002).

There are differences in the dominance of AOB and NOB. AOB are generally believed to be dominated over NOB with a ratio of 2.0–3.5 under conditions of good nitrification (You et al., 2003). However, another study revealed that the population of AOB was lower than that of NOB. Schramm et al. (Schramm et al., 1999) found that NOB (*Nitrospira*) was 30 times more abundant than AOB (*Nitrosospira*) in a wastewater treatment plant. This difference between bacterial populations and their functions could be due to different environments within the treatment systems, or could be affected by the precise genomic content of the bacterial strains (Logan et al., 1998; Liu et al., 2013).

In this study, ammonium is oxidized to nitrite by AOB. Partial nitritation process avoids formation of nitrate, thereby reducing oxygen requirements by up to 50%. However, in order to achieve NH_4^+ partial nitrification, nitrite oxidizing bacteria (NOB)

must be inhibited (Hellinga et al., 1998). This can be achieved by selective washout of NOB at high free ammonia (FA) concentrations or low dissolved oxygen (DO) concentrations (Anthonisen et al., 1976). NOB was inhibited by NH_4^+-N in the range of 0.1–1.0 mg/L, while AOB can tolerate NH_4^+-N as high as 10–150 mg/L (Yang et al., 2007). Since AOB have a lower half-saturation coefficient for oxygen than NOB, they generally outcompete NOB at low DO concentrations (Sliekers et al., 2005). The purpose of this study was to investigate the microbial ecology in partial nitritation system for controlling the NO_2^--N/NH_4^+-N ratio of 1:1 in effluent to the subsequent ANAMMOX autotrophic nitrogen removal process.

MATERIALS AND METHODS

Experimental reactor and sample collection

A plug-flow anoxic/aerobic (A/O) process was used to control the reaction between high-ammonia wastewater and sewage (Fig. 1).

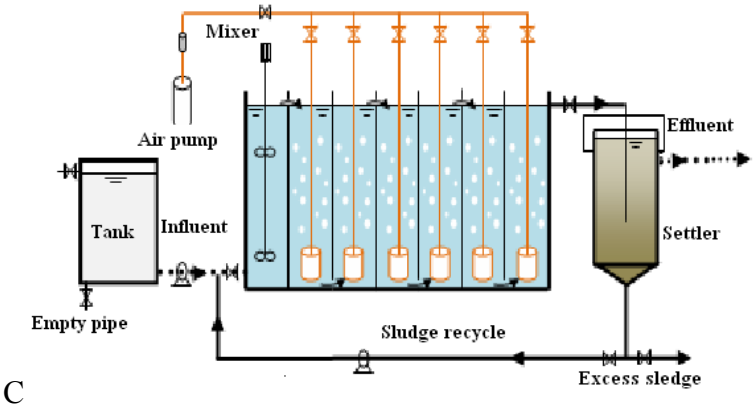

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Fig. 1. The schematic diagram of plug-flow nitrogen removal process.

The nitritation reactor has a working volume of 28 L. Baffles were used to divide it into 7 equal chambers. The first chamber was the anoxic zone, and the other 5 chambers were the aerobic zones. Influent flow and sludge recycle flow rate were controlled through a peristaltic pump. The sewage system was aerated with the sand head, and the high-ammonia wastewater system was aerated with the microporous aeration tube; aeration rate was measured using a rotameter. To achieve good mass transfer, activated sludge and sewage were mixed through the blender in the anoxic zone. A/O reactor operating temperature was controlled at 26 ± 1°C by using a heating rod. The running conditions of the A/O reactor and the influent quality and running effect of A/O reactor were as shown in Table 1.

In the present study, the residual sludge from a high-ammonia system (containing a large number of AOB) was added into the sewage system, through adjusting the concentration ratio of DO and hydraulic retention time (HRT) to achieve partial nitritation of sewage. Activated sludge was collected from the last tank. All collected samples were immediately transported to the laboratory for analysis.

Table 1 (a) The running conditions of the A/O reactor

System	Q (L/d)	HRT (h)	SRT (d)	DO
Sewage system	82	8.2	10	0.41
High-ammonia system	184	3.13	4.5	0.96

Note: Influent rate (Q), hydraulic retention time (HRT), sludge retention time (SRT), dissolved oxygen of the aerobic zones (DO)

Table 1 (b) The influent quality and running effect of the A/O reactor

System	Influent ammonia (mg/L)	Influent COD (mg/L)	MLSS (mg/L)	SVI (mg/L)	NAR (%)
Sewage system	42.08~53.16 (47.70)	121.0~165.7 (142.6)	3500~5000 (4300)	74~77 (75)	84.59~93.99 (90.60)
High-ammonia system	291~387 (337)	100~171 (128)	2500~3500 (3064)	52~95 (77)	95.0%~98.3 (96.6)

Note: Average value in brackets, mixed liquid suspended solids (MLSS), sludge volume index (SVI), nitrite accumulation rate (NAR)

PLFA Analysis

Composite PLFA profiles are an indication of microbial biomass (Ben-David et al., 2004) and provide information on the community structure, and how the microbial community system changes according to the amount of ammonia present.

The 15 mL sample and 20 mL chloroform, methanol, and citrate buffer (Bligh-Dyer extraction, pH 7.4) (1:2:0.8, v/v/v) were mixed in a 50-mL centrifuge tube. The mixture were shaken in the dark for approximately 2 h, and then left to stand overnight. Next, the chloroform and citrate buffer (1:1, v/v) were added in order to achieve phase separation. The lower lipid phase was removed and dried under a stream of N_2, and the lipid fraction containing the phospholipids was isolated and translated into fatty acid methyl esters (FAMEs) by using a mild alkaline methanolysis reaction (Guckert et al., 1985). The samples were stored at -20°C for future analysis. FAMEs were analyzed using gas

chromatography-mass spectrometry (GC–MS; Hewlett–Packard 6890 Gas Chromatograph with HP5973 Mass Spectrometer). Nonacosane acid methyl ester (19:0) was used as a quantitative internal standard of known concentration. The fatty acids were quantified by comparing the peak areas with those of the standard peak. Fatty acid terminology utilizes "A:BωC," where "A" indicates the total number of carbon atoms, "B" is the number of unsaturated carbons, and "ω" precedes "C," the number of C atoms between the closest unsaturated atom and the aliphatic end of the molecule. The suffixes *c* and *t* indicate cis and trans geometric isomers, respectively. The prefixes *i* and *a* refer to iso and anti-iso methyl branching, respectively, and *10Me* refers to a methyl group on the tenth carbon from the carboxylic end of the fatty acid. All data are represented in percent (w/w) of total FAME. The following fatty acids i14:0, i15:0, a15:0, i16:0, 16:1ω7c, i17:0, 17:1ω6c, a17:0, 17:0cy, 18:1ω7c, 18:1ω5c, and 19:0cy were chosen to represent the PLFAs of the bacterial group and fungal 18:2ω6c (Tunlid et al., 1989). Principal component analysis (PCA) was performed to compare the PLFA profiles in the samples by using SPSS10 software (version 13.0, SPSS Inc).

DNA extraction

Nucleic acids were extracted from 200 μL fresh mixed liquor using a Tanbead Activated Sludge DNA Auto plate (Taiwan) according to the manufacturer's protocol. DNA was stored at -20°C before use.

Real-time PCR assay

Real-time PCR was performed on an M×3000P fluorescent quantitative PCR thermocycler (Genetimes). Amplification reactions were carried out using the SYBR Green PCR master mix (Tiangen, China). The 16S rRNA gene of AOB and NOB and bacterial *amoA* genes were quantified in a 25 μL reaction mixture, including the primers concentration of 200 nM, DNA template volume of 1 μL, PCR master mix of 11.25 μL, and sterilized ultrapure water. Primers amoA-1F/amoA-2R (Rotthauwe et al., 1997), CTO-189F/CTO-654R (Kowalchuk et al., 1997), and NSR 1113F/NSR 1264R (Dionisi et al., 2002) were used for the quantification of the bacterial amoA genes, AOB 16S rRNA gene, and NOB 16S rRNA gene, respectively (Table 2). The real-time PCR thermocycling steps for all primer sets were as follows: 95°C for 10 min, and 40 cycles at 95°C for 45 s, 56°C for 45 s, and 72°C for 1 min (Suzuki et al., 2000).

Table 2 Primers used for the PCR amplification

Target group	Primer	Sequence (5'-3')	Length of amplicon (bp)
amoA	amoA-1F	GGGGTTTCTACTGGTGGT	490
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC	
AOB	CTO-189F	GGAGMAAAGYAGGGGATCG	465
	CTO-654R	CTAGCYTTGTAGTTTCAAACGC	
	F338	CCTACGGGAGGCAGCAG	200
	R518	ATTACCGCGGCTGCTGG	
NOB	NSR 1113F	CCTGCTTTCAGTTGCTACCG	151
	NSR 1264R	GTTTGCAGCGCTTTGTACCG	

In order to obtain accurate results, in all experiments, appropriate negative controls containing no template DNA were subjected to the same procedure to exclude or detect any possible contamination. The melting curves were also routinely checked to confirm purity of the amplified products. The parameter Ct (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected. Standard curves for real-time PCR assays were developed as described previously (He et al., 2007). Briefly, the bacterial *amoA* gene, AOB, and NOB 16S rRNA gene were PCR-amplified from extracted DNA with the primers amoA1F/amoA-2R′, CTO-189F/CTO-654R, and NSR1113F/NSR1264R, respectively, and the PCR products were cloned into the pGEM-T Easy Vector (Promega). Plasmids used as standards for quantitative analyses were extracted from the correct insert clones of each target gene. The plasmid DNA concentration was determined on a Nanodrop ND-2000 (Thermo Technologies), and the copy numbers of target genes were calculated directly from the concentration of the extracted plasmid DNA. Nine-fold serial dilutions of a known copy number of the plasmid DNA were subjected to real-time PCR assay in triplicate to generate an external standard curve.

PCR-DGGE Fingerprinting Analysis

For DGGE analysis, the primers used for AOB 16S rRNA gene amplification were CTO-189F/CTO-654R, and the primers used for the second amplification were F338GC/R518 (Shabir et al., 2005). The variable V3 region of 16S rRNA gene sequences from nucleotide 338 to nucleotide 518 (*Escherichia coli* numbering) was amplified, and the GC-clamp was attached to the F338 primer to prevent the separation of the DNA fragments on the DGGE gel. DGGE was performed with the DcodeTM universal mutation detection system (Bio-Rad, USA). PCR products (50 μL) were loaded onto 10% (w/v) acrylamide:bisacrylamide (37.5:1, m/m) gel containing a 40%–60% linear gradient of formamide and urea (100% denaturing solution contained 40% formamide and 7 mol/L

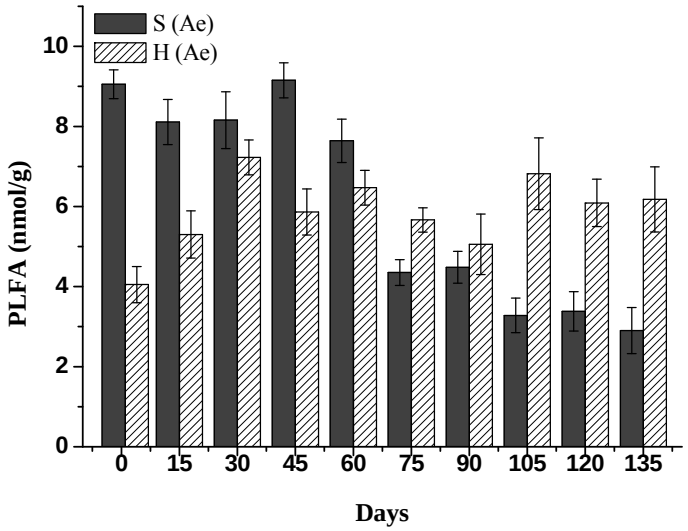
urea). The gel was run for 12 h at 60°C and 80 V in 1 × TAE buffer. After electrophoresis, the gel was stained with 0.5 μg/mL ethidium bromide and photographed with a Fluor-S Multi Imager System (Bio-Rad, USA). DGGE bands of interest were excised from the gel using a sterile scalpel. The DNA was subsequently eluted overnight at 37°C in sterilized distilled water. Excised DNA was then re-amplified using the primer F338/R518 as described previously. The purified PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5*α*. The inserted fragments of 5 positive clones randomly selected from each clone library (16 libraries in total) were sent to Bomaide Biological Engineering Technology & Services Co., Ltd. (Beijing, China) for DNA sequencing. The sequences were compared with those stored in GenBank using BLAST algorithm multiple alignments, and distance matrix analyses were conducted using the MEGA 4.1 software package. A phylogenetic tree was constructed using neighbor-joining analysis with 1000 replicates of bootstrap analysis.

RESULTS

PLFA analyses

The analysis of aerobic and anaerobic biomass of phospholipids and fatty acids showed that aerobic microorganisms were the dominant bacteria in communities; their biomass was higher than that of anaerobic bacteria, which likely included AOB and NOB. The biomass of aerobic bacterial PLFAs significantly decreased with running time in sewage; however, there was little increase with running time in wastewater containing high ammonia. The biomass of anaerobic bacterial PLFAs did not change significantly in high-ammonia wastewater, but did significantly increased with running time in sewage.

(a)



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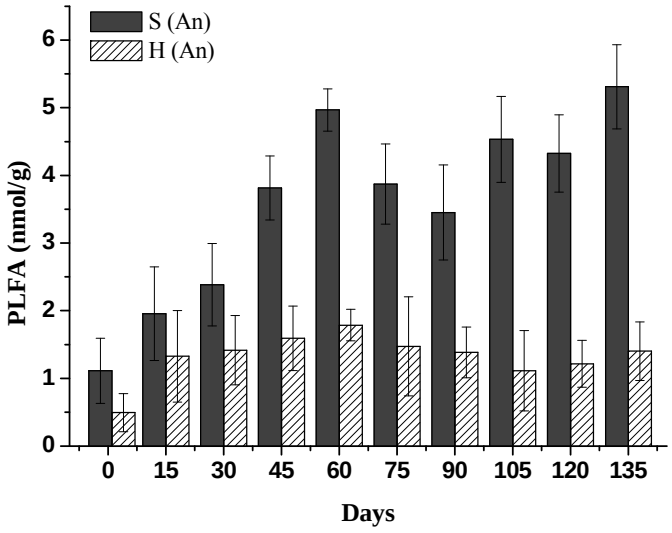
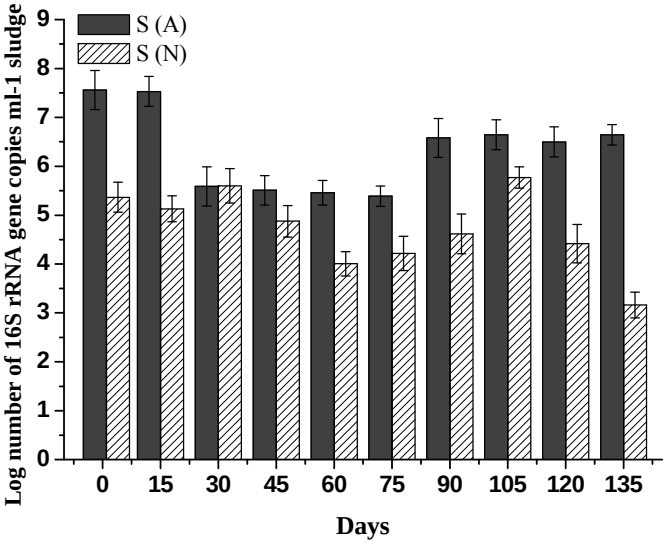


Fig. 2. Response of sewage and high-ammonia wastewater microorganisms. Aerobic bacterial PLFA (a) and anaerobic bacterial PLFA (b). Error bars represent the SE of the mean (n = 3). Aerobic biomass and anaerobic biomass of sewage and high-ammonia wastewater were represented with S (Ae), S (An), H (Ae), and H (An).

Abundance of bacteria in the sludge

The real-time PCR assays suggested that the abundance of AOB and NOB in high-ammonia wastewater ranging from 1.23×10^7 to 8.95×10^8 copies/mL mixed liquor and 1.11×10^4 to 2.47×10^6 copies/mL mixed liquor, respectively, were significantly higher than those in sewage, which ranged from 4.19×10^5 to 3.95×10^7 copies/mL mixed liquor and 1.46×10^3 to 5.89×10^5 copies/mL mixed liquor, respectively (Fig. 3).

(a)



(b)

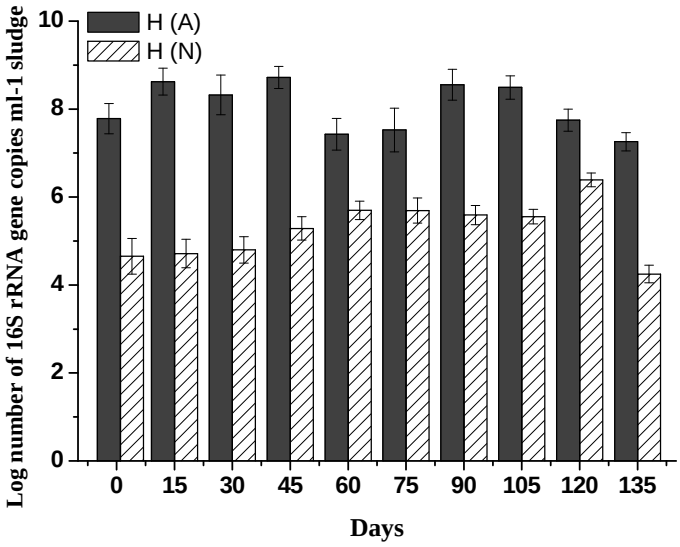


Fig. 3 AOB and NOB 16S rRNA genes copy numbers in sewage (a) and high-ammonia wastewater (b). Sewage: AOB S (A), NOB S (N). High-ammonia wastewater: AOB H (A), NOB H (N).

In sewage (Fig.3a), the abundance of NOB was decrease with most of the running time, especially the last samples contained a very low amount of NOB in comparison to the other sludge specimens; this is consistent with the lack of nitrite oxidation in this sludge type (Pynaert et al., 2002).

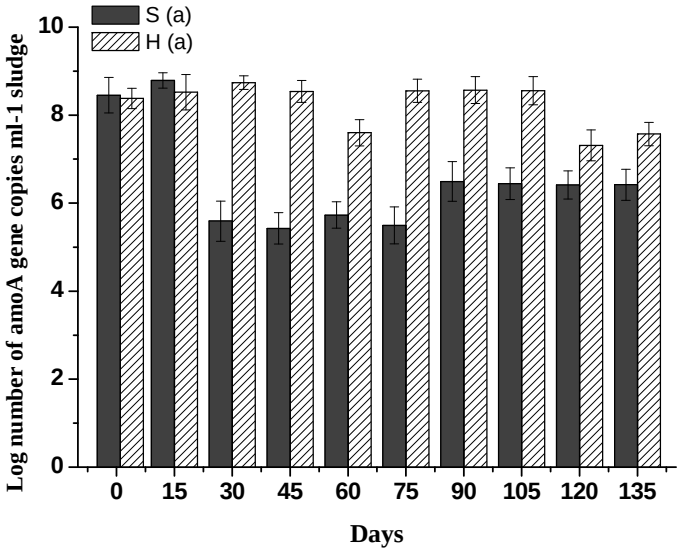


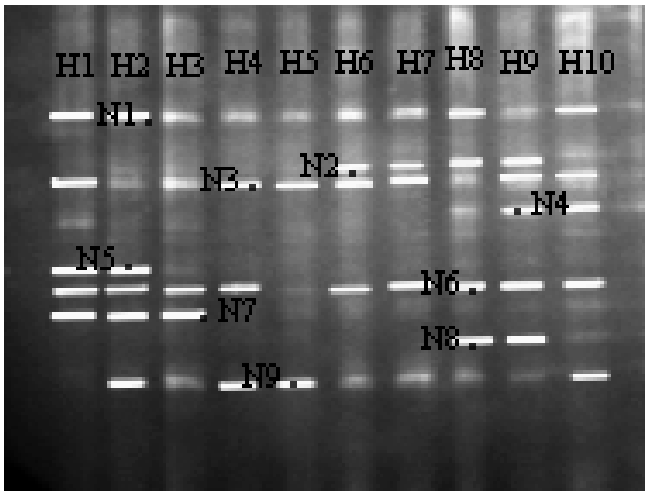
Fig. 4. *amoA* gene copy numbers in sewage and high-ammonia wastewater. Sewage: S (a), High-ammonia wastewater: H (a)

Abundance of AOB was estimated by quantifying their respective *amoA* gene copy numbers (Fig. 4). The highest bacterial *amoA* gene copy numbers at 7.99×10^9 copies/mL mixed liquor were found in high-ammonia wastewater, and the lowest appeared in the sewage (2.30×10^5), where the bacterial *amoA* gene copy number significantly increased concomitant with increased ammonia concentration. Significant variations were found in the sewage, where the *amoA* gene copy number was high at the beginning, decreased significantly by the third sample, and then again gradually increased. In contrast to the situation in sewage, there were little differences in *amoA* gene copy number in high-ammonia wastewater. The *amoA* gene copy number in sewage, ranging from 1.24×10^5 to 4.90×10^8 mL^{-1} of sludge, was lower than that in high-ammonia wastewater, which ranged between 7.43×10^6 and 7.99×10^9 mL^{-1} of sludge. In addition, there was no significant difference in initial *amoA* gene copy number between sewage and high-ammonia wastewater.

PCR-DGGE Fingerprinting Analysis

The community structure of AOB was analyzed by DGGE, and the resulting profiles from high-ammonia wastewater and sewage are shown in Fig. 5. Bands with the same mobility in the DGGE gels were marked with the same number and excised for phylogenetic analysis. DGGE patterns of AOB clearly varied among different samples; high-ammonia wastewater and sewage yielded 9 and 7 discrete bands, respectively (Fig. 4). The samples from high-ammonia wastewater had a more diverse banding pattern than those from sewage.

(a)



(b)

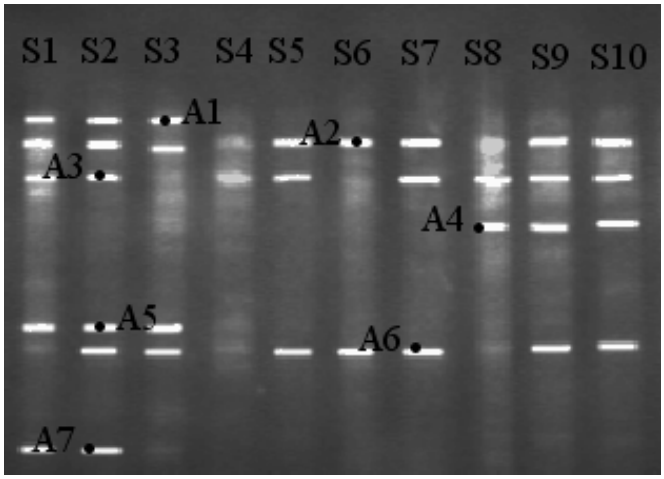
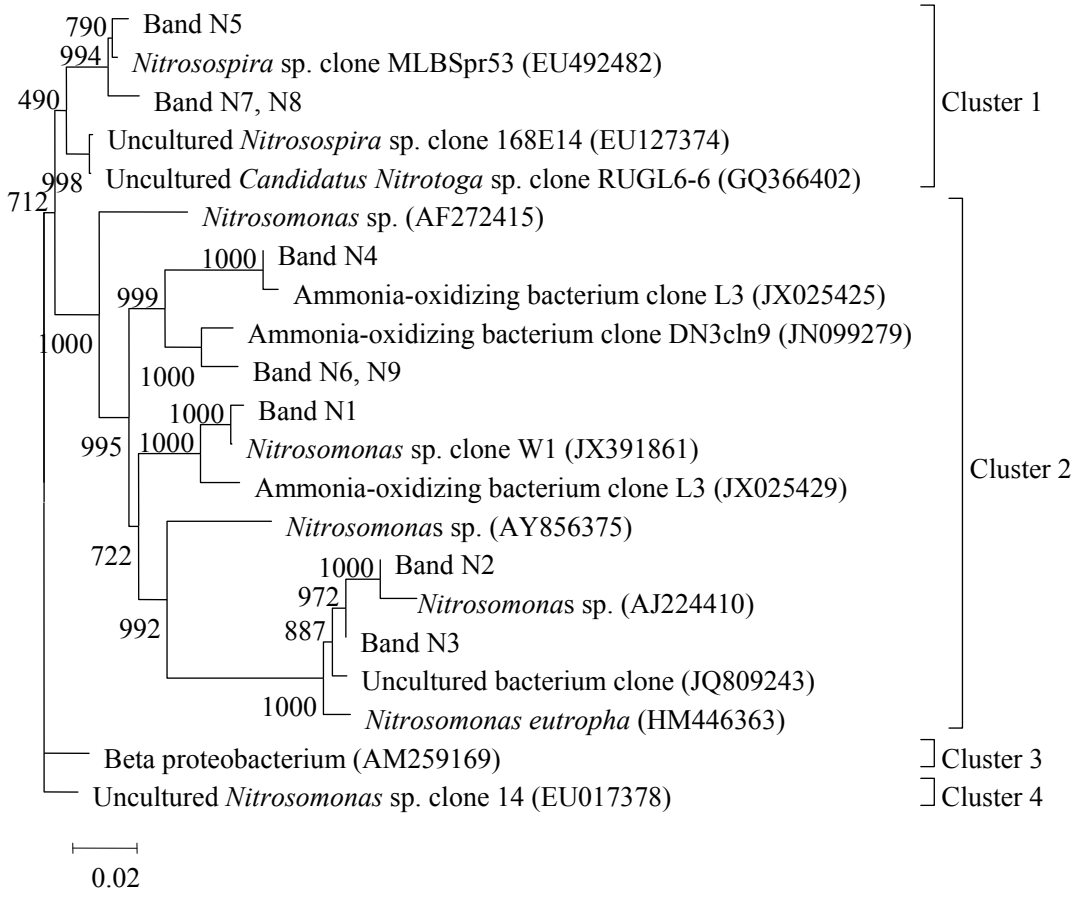


Fig. 5. Denaturing gradient gel electrophoresis profiles of AOB in high-ammonia wastewater (a) and sewage (b). Band position was highlighted with a numbered point.

(a)



(b)

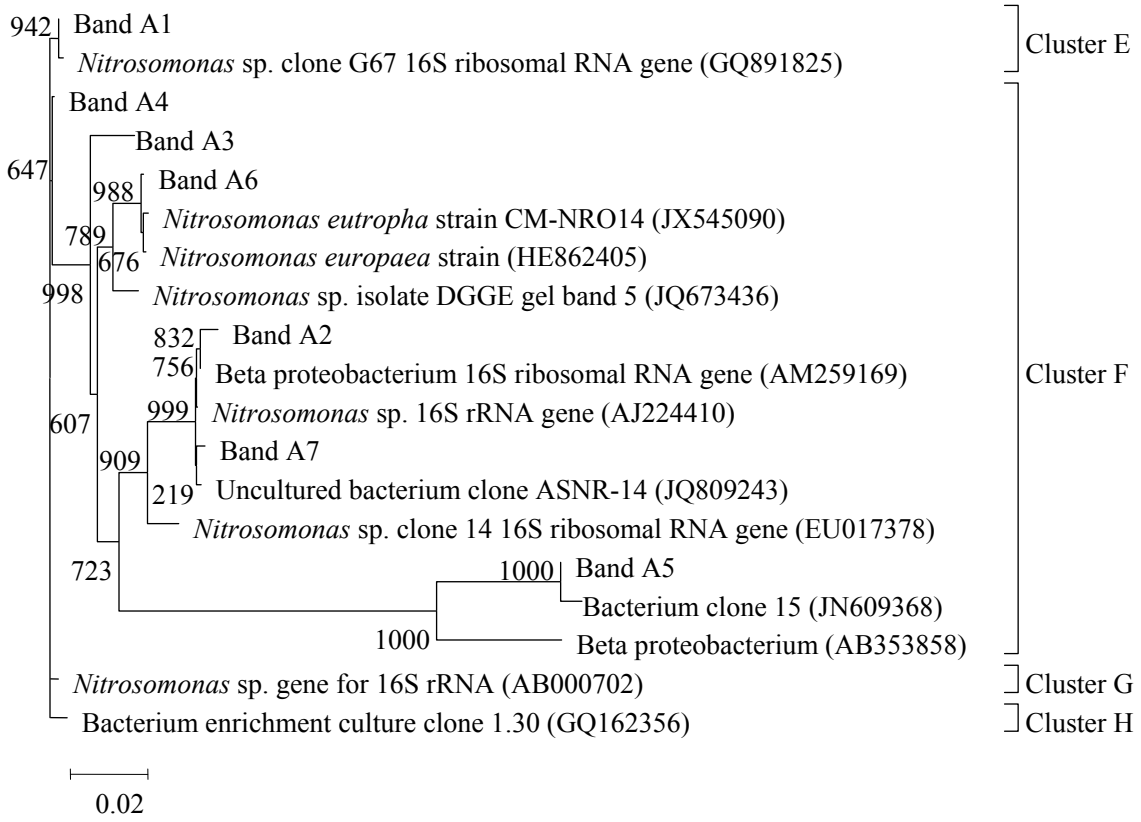


Fig. 6. Phylogenetic relationships among AOB sequences of high-ammonia wastewater (a) and sewage (b). Designation of the clones in bold includes the following information: accession number in the GenBank with DGGE band position. Bootstrap values (>50%) are indicated at branch points. The scale bar represents 5% estimated sequence divergence.

In high-ammonia wastewater, although some bands (N2, N4, N8, N9) were present in the profiles of Fig. 5 (a), their intensity varied. Some bands (N5, N7, N8) disappeared at many of the samples. Bands N1, N3, and N6 had a high intensity in all almost samples. In sewage, band A4 was present in the sample S8, S9, S10 profiles of Fig. 4 (b); some bands (A1, A5, A7) disappeared at many of the samples. Bands A2, A3 and A6 had a high intensity in almost all samples.

The classification for AOB clusters was defined in previous studies (Avrahami et al., 2003). The sequences were used to construct phylogenetic trees with representative sequences retrieved from the GenBank database (Fig. 6). In high-ammonia wastewater (Fig. 6a), all the sequenced clones were belonged to the genuses *Nitrosomonas*-like and *Nitrosospira.* Bands N5, N7, and N8 detected in the first samples were affiliated with *Nitrosospira* sp. (cluster 1). Bands N1, N2, N3, N4, N6, and N9 were grouped in *Nitrosomonas* cluster 2. In addition, bands N2 and N3 were affiliated with the *Nitrosomonas eutropha*. Phylogenetic analysis of the 16S rRNA genes in sewage bacteria

showed that all selected clones could be divided into 2 clusters, i.e., cluster E and cluster F (Fig. 6b). The majority of AOB sequences were grouped in cluster F, revealing their low diversity. All the bands were affiliated with genus *Nitrosomonas*.

DISCUSSION

In this study, PLFA analysis was used to determine the microbial biomass (Fig. 2). Aerobic bacteria biomass remained stable in high-ammonia wastewater, but significantly decreased in sewage, indicating a high ammonia concentration improved the stability of aerobic bacteria. The proportion of anaerobic bacteria increased in sewage, likely due to the DO concentration less than 0.5 mg/L, which favors anaerobic bacteria growth. However, the level of anaerobic bacteria did not significantly change in high-ammonia wastewater, which likely due to the DO concentration (0.96 mg/L) was sufficiently high to limit anaerobic bacteria growth. Thus, DO concentration also was a factor to affect the partial nitritation process (Wang et al., 2008).

Real-time PCR was used to quantify the 16S rRNA and *amoA* genes copy numbers in high-ammonia wastewater and sewage. High ratios of AOB to NOB were found in all the samples, providing evidence of their potential importance in nitritation. Ammonia concentration was positively correlated with the bacterial 16S rRNA and *amoA* genes copy numbers, indicating ammonia was an important factor in controlling AOB and NOB abundance in the wastewater treatment. More copy numbers of the bacterial *amoA* genes in high-ammonia wastewater were found compared with the sewage. In this study, the inhibition of NOB was found to be critical for partial nitritation, since NOB oxidize NO_2^- to NO_3^- and cause a shift from partial nitritation to complete nitrification. Therefore, the DO is likely a key factor that determines AOB and NOB abundance in wastewater treatment (Freitag et al., 2003). Although AOB abundance varied between different samples, these bacteria were only a small proportion of the total bacterial population. However, AOB are a more sensitive indicator of the nitrogen removal process in wastewater treatment than the total bacteria (Kowalchuk et al., 2001). There was a significant correlation between ammonia concentration and AOB abundance, suggesting that AOB abundance could be predicted by ammonia (Bernhard et al., 2007). Little correlation between ammonia concentration and NOB abundance was observed among the different treatments, suggesting that ammonia concentration may not be a substantial determinant of NOB community levels during partial nitritation.

Polymerase chain reaction-DGGE method was used to analyze the community structure of AOB 16S rRNA genes. There was a significantly positive correlation between AOB abundance and ammonia concentration, which implied that the change of AOB abundance might be partly due to the shifts of the community composition. Moreover, the DGGE patterns from the high-ammonia wastewater samples showed an increase in the diversity of the AOB community. Addition of sewage decreased the diversity of AOB in all the samples. Horz et al. (2004) also reported a significant change in the structure of

AOB communities after nitrogen concentration was increased. Thus, AOB community composition is consistently altered by nitrogen concentration, and particularly by elevated ammonia levels, and this affects the potential for ammonia oxidation. There was no difference in the AOB community structure in sewage samples. However, a pronounced difference in AOB composition was observed in high-ammonia wastewater. It is likely that the AOB community in sewage is not as sensitive as to changes in nitrogen in comparison to that in high-ammonia wastewater.

The phylogeny of the 16S rRNA genes was found to correspond largely to the *amoA* genes of AOB (Aakra et al., 2001). Phylogenetic analysis of bacterial 16S rRNA gene sequences suggested that the AOB in high-ammonia wastewater was dominated by *Nitrosomonas* and *Nitrosospira*-like sequences. Most sequences in sewage were affiliated with *Nitrosomonas* cluster F, whereas *Nitrosospira*-like sequences were not present. This is consistent with observations that *Nitrosomonas*-like bacteria were detected as the dominant ammonia-oxidizer group in many low DO wastewater samples (Schramm et al., 2000; Blackburne et al., 2007b). *Nitrosomonas* species have been detected in partial nitritation systems (Logemann et al., 1998), providing further evidence of their preference for ammonia concentration.

This study showed significantly higher copy numbers of the AOB 16S rRNA genes in high-ammonia wastewater compared to that in sewage. PLFA and DGGE patterns also revealed community shifts among the different ammonia concentration samples. These results are consistent with many studies that show AOB provide a good index in response to the varying environment. Thus, we hypothesize that ammonia concentration is a key factor in stimulating AOB community change, and further studies should be performed to determine other characteristics that affect the activity and structure of AOB.

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